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Contribution of Penicillin-Binding Protein Homologs to Antibiotic Resistance, Cell Morphology, and Virulence of *Listeria monocytogenes* EGDe

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Seven open reading frames, annotated as potential penicillin-binding-protein-encoding genes (lmo0441, lmo0540, lmo1438, lmo1892, lmo2039, lmo2229, and lmo2754), were targeted for insertional mutagenesis in *Listeria monocytogenes* EGDe. These genes were found to contribute in various degrees to β-lactam resistance, cell morphology, or the virulence potential of this organism.

Listeria monocytogenes is an important intracellular pathogen that is responsible for almost 30% of food-related deaths in the United States every year (19). In general, *L. monocytogenes* is susceptible to a wide range of antibiotics, including penicillins, aminopenicillins, and carbapenems (5, 11). Clinicians treating patients suspected of having *L. monocytogenes* infection normally utilize ampicillin or benzylpenicillin, either alone or in combination with an aminoglycoside (30). Although rare, there have been some reports of penicillin-resistant *Listeria* isolated from foods (35), and ampicillin-resistant and imipenem-resistant clinical isolates have also been reported (24, 26). *L. monocytogenes* strains also possess an innate natural resistance to monobactams, β-lactams such as methicillin, and the expanded-spectrum cephalosporins, including cefotaxime and ceftazidime (5). The last of these traits is of clinical significance, as the cephalosporin antibiotics are often the first therapy used to treat fever in hospitals when the etiological agent is unknown.

To date there have been a limited number of studies pertaining to the penicillin-binding proteins (PBP) of *L. monocytogenes*, and, significantly, the majority of these were carried out before sequencing of a *L. monocytogenes* genome was completed (8, 9, 10, 22, 32, 33). Recent attempts have been made to reevaluate the molecular weights and cell copy numbers of PBPs in *L. monocytogenes* EGD (15), and the enzymatic properties of PBP4 (encoded by lmo2229) (36) and PBP5 (encoded by lmo2754) (16, 17) have been established. In this study potential PBP-encoding genes of the genome-sequenced EGDe strain were found through in silico analysis, and subsequently their respective roles were revealed by insertional mutagenesis.

Putative PBPs of *L. monocytogenes* EGDe were identified using the free text function on the genome web server Listilist (http://genolist.pasteur.fr/listilist/). Subsequently sequences were analyzed using various web-based programs (www.ncbi.nlm.nih.gov/structure/cdd/cddshtml, www.ncbi.nlm.nih.gov/BLAST, www.ncbi.nlm.nih.gov/sutlis/blink, www.tigr.org, www.microbesonline.org, www.ebi.ac.uk/fasta33, www.sanger.ac.uk/software/pfam, and www.ebi.ac.uk/Interpro). The possible functions and closely related homologs of the putative PBPs are outlined in Table 1. Five genes encode >50-kDa high-molecular-mass PBPs (lmo0441, lmo1438, lmo2039 [class B] and lmo1892 and lmo2229 [class A]). Lmo0540 and Lmo2754 (PBP5) are annotated as low-molecular-mass PBPs in the EGDe genome. However, BLAST and domain analyses of Lmo0540 indicate that it shows highest similarities to class C β-lactamases. Also, sequence alignments revealed some slight variations in the positioning of the classical PBP motifs (6). Lmo0540 was nonetheless included in this study to determine its role in *L. monocytogenes* physiology.

Insertional mutagenesis of all seven genes was attempted by the method previously described (3, 18). Primers amplifying central internal fragments of each of the following genes were used for disruption (all sequences are 5′ to 3′): lmo0441 (forward, ACGAATTCGAAATGCGGAC; reverse, TTTCTAGATGTCTTCGGCG), lmo0540 (forward, GATCTAGAACCAGTAGAAG; reverse, GTGAATTCTGGTCGTCCAAC), lmo1438 (forward, CGGAATTCCAATTTGTTGG; reverse, TTTCTAGACCTTCTTTAGC), lmo1892 (forward, AATCTAGAAACGGAAGATGCG; reverse, GCGAATTCCTGTAGTGATAG), lmo2039 (forward, AAGTCGAATTCGGCGCTGCT; reverse, GATCTGCAGGGTTGAAGGA), lmo2229 (forward, AACTGCAGTAGTTTCCATTG; reverse, TGTAGAATTCGCCTTCTGC), and lmo2754 (forward, TCTGAATTCGCTACAAAG; reverse, CGTCTAGACCTTTGCGCC). In each case integration was confirmed by PCR using one primer outside the region of integration and another for the plasmid and was indicated by an Em^r Cm^s phenotype. With the exception of lmo2039, all of the genes were successfully disrupted. lmo2039 is not followed by a termination signal and is located within a putative operon, and thus our inability to isolate an integrant could theoretically be due to a polar impact on this operon. However, as two homologs, PBP2X (36% identity) and PBP1 (37% identity), have been found to be essential for the viability of *Streptococcus pneumoniae* (13) and *Staphylococcus aureus* (34), respectively, it is more likely that the consequences of disrupting lmo2039 itself are the reasons why this mutant was not isolated.

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TABLE 1. Bioinformatic analysis of putative PBPs of *L. monocytogenes*[a]

Gene	Class	No. of amino acids	Predicted mol wt, 10^3	Putative function	Homolog(s)	Reference
lmo0441	B	678	74.6	Carboxypeptidase Transpeptidase	*Enterococcus faecalis* PBP4 *Enterococcus faecium* PBP5 *Staphylococcus aureus* PBP2′	21 38 12
lmo0540	LMW[b]	397	44.5	β-Lactamase	*Staphylococcus aureus fmtA*	14
lmo1438	B	721	79.9	FtsI, cell division Transpeptidase	*Staphylococcus aureus* PBP3	23
lmo1892	A	827	90.8	Carboxypeptidase Glycosyltransferase Transpeptidase	*Streptococcus pneumoniae* PBP1A *Enterococcus faecalis* PBP1A	28 4
lmo2039	B	751	81.8	FtsI cell division Transpeptidase	*Streptococcus pneumoniae* PBP2X *Staphylococcus aureus* PBP1	28 34
lmo2229[c]	A	714	77.8	Carboxypeptidase Glycosyltransferase Transpeptidase	*Streptococcus pneumoniae* PBP2A	28
lmo2754[d]	LMW	445	48.08	Carboxypeptidase Transpeptidase	*Bacillus subtilis* PBP5 *Streptococcus pneumoniae* PBP3	31 28

[a] Gene names, predicted molecular weights and putative functions are based on those assigned by the National Center for Biotechnology Information and by Listilist. Homologs were found by www.ncbi.nih.nlm.gov/blink.
[b] LMW, low molecular weight.
[c] Characteristics of lmo2229 are as outlined previously (36).
[d] Characteristics of lmo2754 are as outlined previously (15, 16).

Before detailed investigations were carried out, the growth rates of the six mutants and the parent strain in tryptic soy broth-yeast extract (TSB-YE) at 37°C were compared (data not shown). While the growth rate of the lmo2754 mutant did differ from that of the parent strain, this difference was not statistically significant as determined by Student's *t* test ($P < 0.05$) (data not shown). To determine if the disrupted PBP-encoding genes play a role in the β-lactam resistance of *L. monocytogenes*, the six mutants were subjected to antibiotic disk assays with a large number of β-lactam disks on tryptone soy agar (Difco) supplemented with 0.6% yeast extract (Merck) (TSA-YE) (data not shown). Following this preliminary assay, specific cephalosporin antibiotics and penicillin G were chosen for MIC determination using the broth dilution (TSB-YE) method outlined by the CLSI (formerly NCCLS) (20) (Table 2). Results were analyzed after 16 to 20 h at 37°C. Both assays demonstrated that interruption of two of the *pbp* homologs, lmo0441 or lmo2229, increased the sensitivity of these strains to β-lactam antibiotics (Table 2). It is interesting to note that for certain antibiotics the disk assays revealed the presence of single colonies of *Listeria* on the outer edges of these zones of clearing, possibly indicating spontaneous resistance development. Of the two, the consequences of mutating lmo0441 were greater as evidenced by a 16-fold reduction in the MICs of ceftazidime and cefotaxime (Table 2). Interestingly, Lmo0441 is homologous to the low-affinity PBPs of *Enterococcus faecium* (PBP5) (44% identity) (38) and *S. aureus* (PBP2′) (34% identity) (12), which contribute greatly to β-lactam resistance in those genera. Although less dramatic, mutation of lmo2229 results in a decrease in the MICs of some cephalosporins against EGDe (Table 2) and *L. monocytogenes* LO28 (unpublished data). The contrast between these results and those of Zawadzka-Skomial et al. (36), who did not observe a difference between the MICs for EGD and a 2229 mutant, may be due to strain variation (EGD versus EGDe/LO28) or to the growth of spontaneously resistant cells over the extended duration used for MIC determination (36). PBP2A of *S. pneumoniae*, a homolog of Lmo2229, has been found to have an important role in the β-lactam resistance of this organism (29, 37). We also investigated the MICs of two other cell-wall-acting antimicrobials, the lantibiotics nisin and lacticin 3147, against the parent and six mutants. Although the

TABLE 2. MICs of penicillin G and cephalosporin antibiotics against EGDe and mutants

Antibiotic[a]	MIC (μg/ml) against:						
	EGDe	pORI::0441	pORI::0540	pORI::1438	pORI::1892	pORI::2229	pORI::2754
P	0.125	0.06	0.125	0.125	0.125	0.06	0.125
CAZ	128	8	128	128	128	32	64
CTX	8	0.5	8	8	8	2	8
CXM	4	0.5	4	4	4	2	4

[a] P, penicillin G; CAZ, ceftazidime; CTX, cefotaxime; CXM, cefuroxime.

MIC of lacticin 3147 was identical in all cases, one mutant, pORI::2229, displayed enhanced nisin sensitivity (1.66-fold decrease in MIC for triplicate experiments; data not shown). This is in agreement with previous findings for mutants of *L. monocytogenes* 412 (7).

The impact of PBP mutations on the virulence of *L. monocytogenes* was investigated by both in vitro and in vivo assays. In vitro assays revealed no significant reduction in the ability of the strains to grow intracellularly in J774 macrophages (not shown), although the lmo2754 mutant strain exhibited a reduced ability (almost a 1-log reduction) to invade C2Bbe1 cells (Fig. 1a). Macrophage assays (27) and invasion assays (2) were performed as previously described. In vivo survival was assayed by intraperitoneal infection of 6- to 8-week-old BALB/c mice and was quantified on the basis of the number of *Listeria* cells in the spleen at 3 days postinfection. Cells were counted on TSA-YE plates incubated overnight at 37°C. A number of mutants displayed dramatic reductions in virulence (Fig. 1b). Here it is demonstrated that in addition to having a role in mouse brain colonization following intravenous inoculation, Lmo0540 also contributes to virulence as determined by colonization of the spleen (1). Disruptions of lmo1438 or lmo2229 also greatly attenuated virulence as demonstrated by almost 2- and 3-log reductions in numbers in the spleen, respectively, while a mutant lacking a functional PBP5 also showed a virulence defect (Fig. 1b).

The impact of the loss of PBP function on the morphology of log-phase *L. monocytogenes* in TSB-YE was initially investigated with a light microscope (magnification of ×1,000). The mutant lacking lmo2229 showed an alteration in morphology, with chains of three or four cells observed (data not shown). As this phenomenon has not been described previously (36), it may indicate that the phenotype is growth phase dependent. Further morphological investigation of all strains was performed by transmission electron microscopy (TEM) on log-phase cells. TEM, in addition to confirming the chaining effect in the lmo2229 mutant, identified a number of additional phenotypes associated with this and other mutants (Fig. 2a to g). The cell lengths were determined for each strain ($n = 30$ to 40 for each strain), except for those cells lacking PBP5, which displayed an irregular morphology (Fig. 2g). Only 3% of parental cells were greater than 2.0 μm in length. This value increased to 29% for the lmo0441 mutant, 27% for the lmo1892 mutant, and 31.8% for the lmo2229 mutant. As observed previously (17), strains lacking PBP5 showed a thicker cell wall (EGDe, 25.12 ± 2.81 nm; PBP5 mutant, 33.68 ± 3.89 nm). It was also noted that 50% of PBP5-lacking cells had an irregular shape (Fig. 2g). This phenotype is not unexpected, as it has previously been documented that the loss of low-molecular-mass PBPs can result in altered cell shape (25). It was reported that the EGDΔPBP5 strain has an altered ratio of pentapeptides to tripeptides (16). This has been suggested to lead to an imbalance of peptidoglycan, resulting in an altered shape. Finally, slight alterations were found with the disruption of lmo0441 (Fig. 2b) and lmo1892 (Fig. 2e).

In this study, we have completed a postgenomic analysis of loci in the EGDe genome that show homology to PBP-encoding genes and have investigated the roles of the individual gene products by insertional mutagenesis. This is the first study that characterizes the PBPs of *L. monocytogenes* at a genetic level and examines their role in the biology of the organism. Other genes, not studied in this paper, including lmo1855 and lmo2812 (both annotated as DD-carboxypeptidases) and lmo1916 (annotated as a peptidase), with molecular masses of approximately 31, 29, and 38 kDa, respectively, may also merit study. Importantly, this study has identified three new potential therapeutic targets for *L. monocytogenes*. First, MIC determinations suggest that Lmo0441 is central to the β-lactam resis-

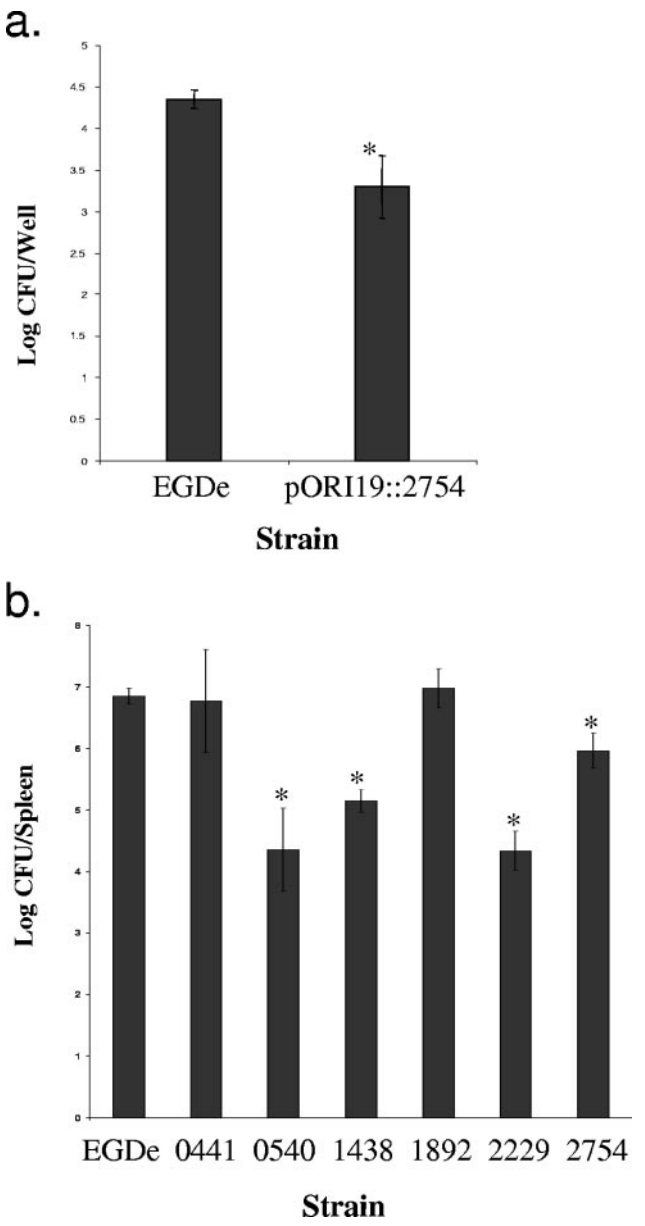

FIG. 1. (a) Effect of disruption of lmo2754 on the invasion of the organism on C2Bbe1 cells. Numbers of *L. monocytogenes* cells invaded were expressed as log CFU/well. Results are representative of triplicate experiments. (b) Effect of pORI19 disruption of PBPs on the survival of EGDe in vivo. Numbers indicate the genes that were disrupted. Mice were injected intraperitoneally, and the number of bacteria recovered from the spleen was determined at 3 days postinfection. Error bars represent the standard deviations from the means ($n = 5$). *, mean is statistically significant with respect to the wild-type value ($P < 0.05$).

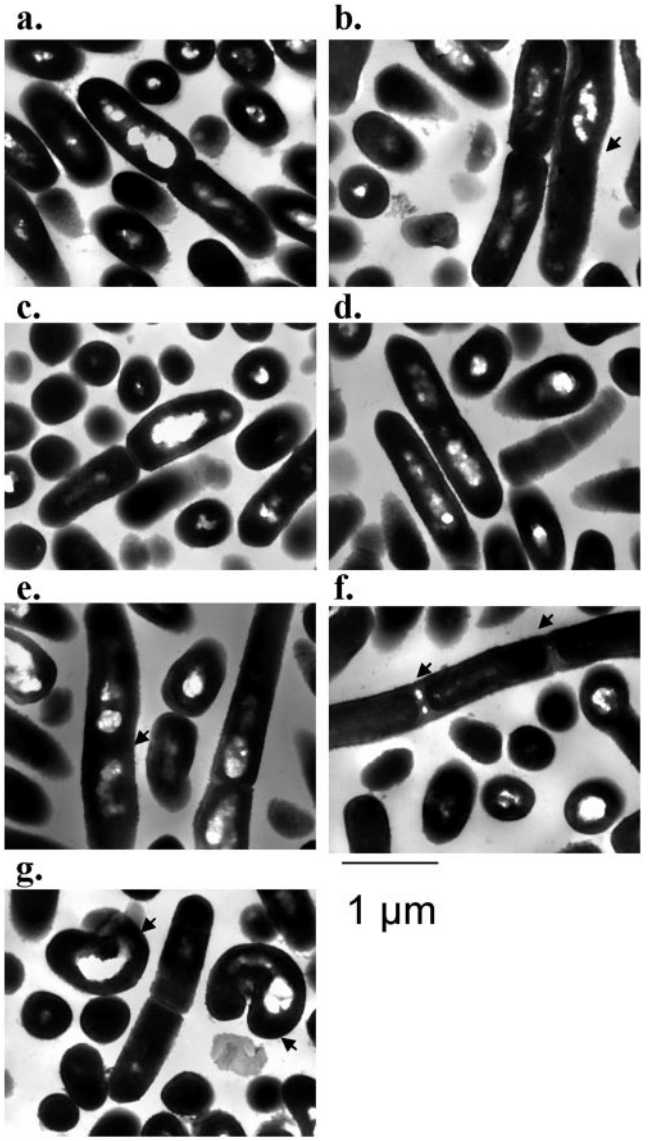

FIG. 2. Electron microscopy of EGDe (a) pORI19::0441 (b), pORI19::0540 (c), pORI19::1438 (d), pORI19::1892 (e), pORI19::2229 (f), and pORI19::2754 (g). Arrows in panels b and e indicate irregular curving of cells and increased cell length. Arrows in panel f indicate filamenting of cells. Arrows in panel g indicate irregular shapes of cells.

tance of this organism, a role which is consistent with its homology to low-affinity PBPs. Second, Lmo2229 contributes to β-lactam resistance, virulence potential, and morphogenesis of *L. monocytogenes*. Being a class A protein, this protein has particular potential as a target due to the presence of a transglycosylase domain. Finally, lmo2039 (or at least the operon in which it resides) is essential to the survival of the organism.

Although *L. monocytogenes* is highly sensitive to a number of antibiotics and at present is treatable with penicillin/ampicillin, the exact mechanism of cell death remains unknown. Also, due to the rapidity with which antibiotic-resistant mutants of other gram-positive bacteria have emerged, it is possible that future alternative therapeutics may be required for the treatment of listeriosis. The characterization of the PBPs of this organism is fundamental to the scientific community's ongoing efforts to limit the mortality associated with this pathogen.

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